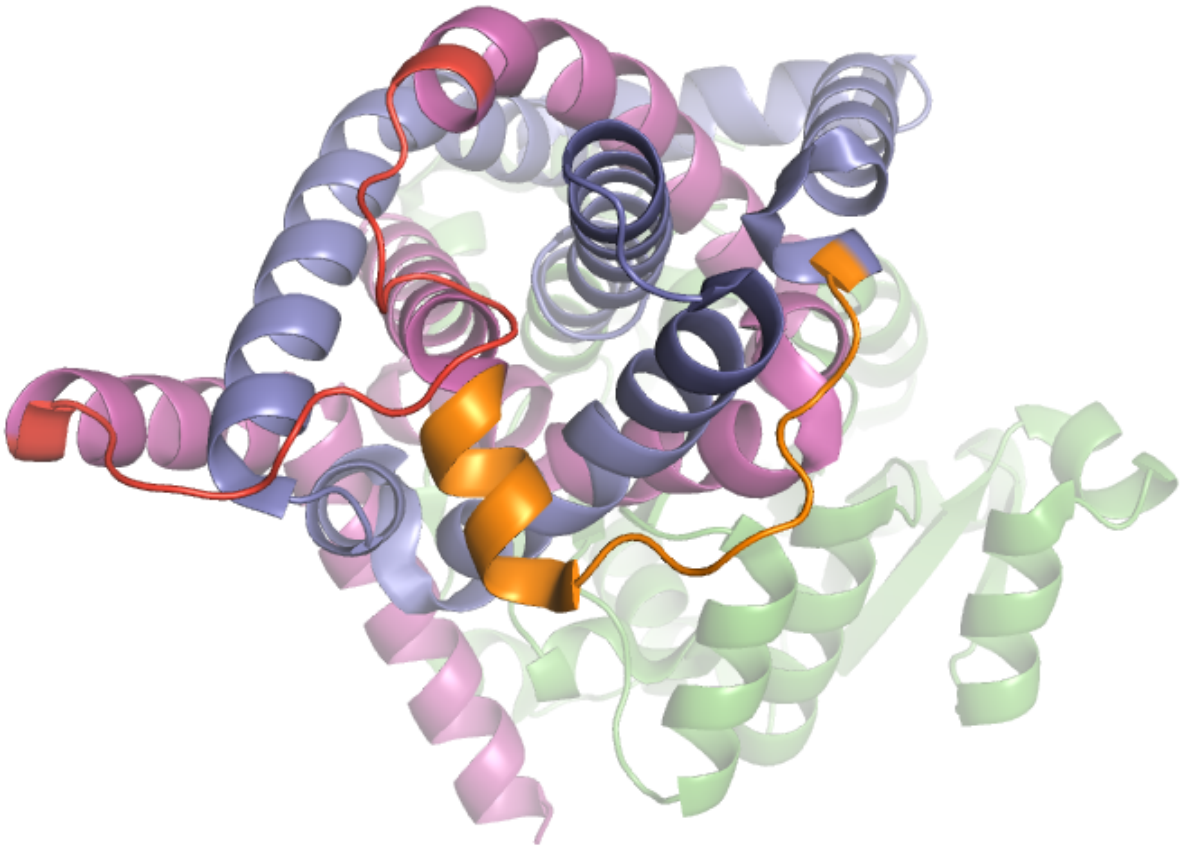
SI 1. Construction of 2Ia_KARI-HT and 2Ia_KARI-2L

Inspection of the Ec_KARI structure revealed that the α1+1-helix was truncated at its N-terminal end with respect to the corresponding α1'-helix of the Ia_KARI dimer. Therefore, in the third construct, 2Ia_KARI-HT (helix truncation), we began the duplicate knotted domain at the first position of this helix which could be aligned to a corresponding position of the Ec_KARI structure, valine 197. As a result, 2Ia_KARI-HT is eight amino acids shorter than 2Ia_KARI-DD.

Comparing the two knotted domains of Ec_KARI, one final structural difference is clear. The second knotted domain of Ec_KARI does not have an α5-helix, which is replaced by the long α4+1α6+1-loop (Supplemental Information 2), and the α6+1 helix is shifted and rotated with respect to an alignment with Ia_KARI. Our fourth and final construct, 2Ia_KARI-2L (two [transplanted] loops), is constructed from 2Ia_KARI-LT by identification of the final positions which can be aligned around this dissymmetry, lysine 264 and glutamate 315 of Ia_KARI, and replacing the 50 residues between them, which compose the C-terminal of the α4'-helix, the α4'α5'-loop, the α5'-helix, the α5'α6'-loop, the α6'-helix, and the α6'α7'-loop, with the 44 residues composing the C-terminal of the α4+1-helix, the α4+1α6+1-loop, the α6+1helix, and the α6+1α7+1 loop of Ec_KARI.

However, neither of these constructs yielded sufficient folded, purifiable protein for characterization.



SI 2. A view of Ec_KARI (PDB 3ULK) showing the two structural deviations from class I KARIs. The Rossmann domain is shown in green, the first knotted domain is shown in purple, and the second is shown in pink. The α6α1+1-loop is shown in orange, connecting the two knotted domains, and the α4+1α6+1-loop is shown in red.

SI 3. Structural nomenclature for KARIs, from Cahn et al.[28]:

Secondary structure elements are numbered starting from the first β-strand that makes up the canonical Rossmann fold; secondary structure prior to this strand is not conserved. In the Rossmann domain, β-strands are numbered 1–8 and α-helices are assigned the letters from A–G. In the knotted domain, which is entirely α-helical, helices are assigned numbers from 1 to 8. Loops between secondary structural elements are named based on the flanking secondary structural elements, as in β2αB-loop or α3α4-loop.

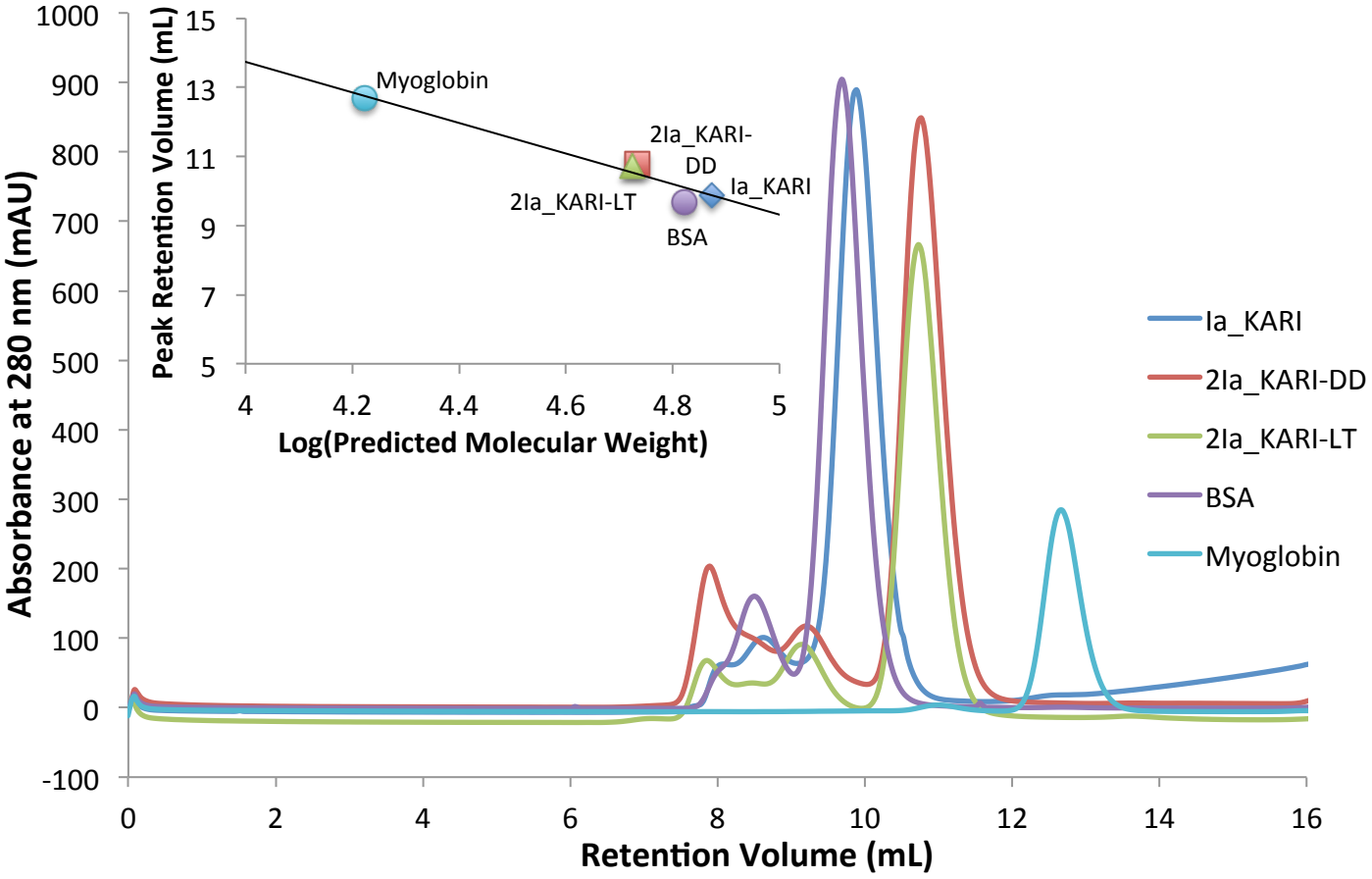
In class I KARIs, the secondary structural elements of the dimeric partner are denoted with a prime as in α3′-helix. In class II KARIs, the duplicated elements are denoted with '+1', as in α3+1-helix.

```
Ia_KARI             ------------------MAKIYKDEDI-SLEPIKNKTIAILGYGSQGRAWALNLRDSGLNVVVGLERQG-----DSWRRAIDD
Ec_KARI             MANYFNTLNLRQQLAQLGKCRFMGRDEFADGASYLQGKKVVIVGCGAQGLNQGLNMRDSGLDISYALRKEAIAEKRASWRKATEN
2Ia_KARI-DD         ------------------MAKIYKDEDI-SLEPIKNKTIAILGYGSQGRAWALNLRDSGLNVVVGLERQG-----DSWRRAIDD
2Ia_KARI-HT         ------------------MAKIYKDEDI-SLEPIKNKTIAILGYGSQGRAWALNLRDSGLNVVVGLERQG-----DSWRRAIDD
2Ia_KARI-LT         ------------------MAKIYKDEDI-SLEPIKNKTIAILGYGSQGRAWALNLRDSGLNVVVGLERQG-----DSWRRAIDD
2Ia_KARI-2L         ------------------MAKIYKDEDI-SLEPIKNKTIAILGYGSQGRAWALNLRDSGLNVVVGLERQG-----DSWRRAIDD

Ia_KARI             GFKPMYTKDAVAIADIIVFLVPDMVQKSLWLNSVKDFMKKGADLVFAHGFNIHFKIIEPPKDSDVYMIAPKSPGPIVRRSYEMGG
Ec_KARI             GFKVGTYEELIPQADLVINLTPDK-QHSDVVRTVQPLMKDGAALGYSHGFNIVEVGEQIRKDITVVMVAPKCPGTEVREEYKRGF
2Ia_KARI-DD         GFKPMYTKDAVAIADIIVFLVPDMVQKSLWLNSVKDFMKKGADLVFAHGFNIHFKIIEPPKDSDVYMIAPKSPGPIVRRSYEMGG
2Ia_KARI-HT         GFKPMYTKDAVAIADIIVFLVPDMVQKSLWLNSVKDFMKKGADLVFAHGFNIHFKIIEPPKDSDVYMIAPKSPGPIVRRSYEMGG
2Ia_KARI-LT         GFKPMYTKDAVAIADIIVFLVPDMVQKSLWLNSVKDFMKKGADLVFAHGFNIHFKIIEPPKDSDVYMIAPKSPGPIVRRSYEMGG
2Ia_KARI-2L         GFKPMYTKDAVAIADIIVFLVPDMVQKSLWLNSVKDFMKKGADLVFAHGFNIHFKIIEPPKDSDVYMIAPKSPGPIVRRSYEMGG

Ia_KARI             GVPALVAVY--QNVSGEALQKALAIAKGIGCARAGVIESTFKEETETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFET
Ec_KARI             GVPTLIAVHPENDPKGEGMAIAKAWAAATGGHRAGVLESSFVAEVKSDLMGEQTILCGMLQAGSLLCFDKLVEEGTDPAYAEKLI
2Ia_KARI-DD         GVPALVAVY--QNVSGEALQKALAIAKGIGCARAGVIESTFKEETETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFET
2Ia_KARI-HT         GVPALVAVY--QNVSGEALQKALAIAKGIGCARAGVIESTFKEETETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFET
2Ia_KARI-LT         GVPALVAVY--QNVSGEALQKALAIAKGIGCARAGVIESTFKEETETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFET
2Ia_KARI-2L         GVPALVAVY--QNVSGEALQKALAIAKGIGCARAGVIESTFKEETETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFET

Ia_KARI             VNELKLIVDLIYEKGLTGMLRAVSDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGST
Ec_KARI             QFGWETITEALKQGGITLMMDRLSNPAKLRAYALSEQ-LKEIMAPLFQKHMDDIISGEFSSGMMADWANDDKKLLTWREETGKTA
2Ia_KARI-DD         VNELKLIVDLIYEKGLTGMLRAVSDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGST
2Ia_KARI-HT         VNELKLIVDLIYEKGLTGMLRAVSDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGST
2Ia_KARI-LT         VNELKLIVDLIYEKGLTGMLRAVSDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEKTA
2Ia_KARI-2L         VNELKLIVDLIYEKGLTGMLRAVSDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEKTA

Ia_KARI             IETVGRKLREMMFRGMKQISSH----------------------------------------------------------------
Ec_KARI             FETAPQYEGKI---GEQEYFDKG-------VLMIAMVKAGVELAFETMVDSGIIEESAYYESLHELPLIANTIARKRLYEMNVVI
2Ia_KARI-DD         IETVGRKLREMMFRGMKQISSHETDLFGEQVILVGGIMELIKASFETLVEEGYQPEVAYFETVNELKLIVDLIYEKGLTGMLRAV
2Ia_KARI-HT         IETVGRKLREMMFRGMKQISSH--------VILVGGIMELIKASFETLVEEGYQPEVAYFETVNELKLIVDLIYEKGLTGMLRAV
2Ia_KARI-LT         FETAPQYEGKI---GEQEYFDKGV------VILVGGIMELIKASFETLVEEGYQPEVAYFETVNELKLIVDLIYEKGLTGMLRAV
2Ia_KARI-2L         FETAPQYEGKI---GEQEYFDKGV------VILVGGIMELIKASFETLVEEGYQPEVAYFETVNELKLIVDLIYEKGLTGMLRAV

Ia_KARI             ----------------------------------------------------------------------------------------
Ec_KARI             SDTAEYGNYLFSYACVPL-----LKPFMAELQPGDLGKA-IPEGAVDNGQLRDVNEAIRSHAIEQVGKKLRGYM-TDMKRIAVAG
2Ia_KARI-DD         SDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGSTIETVGRKLREMMFRGMKQISSH-
2Ia_KARI-HT         SDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGSTIETVGRKLREMMFRGMKQISSH-
2Ia_KARI-LT         SDTAKYGGITVGKFIIDKSVRDKMKIVLERIRSGEFAREWIKEYERGMPTVFKELSELEGSTIETVGRKLREMMFRGMKQISSH-
2Ia_KARI-2L         SDTAKYGGITVGKACVPL-----LKPFMAELQPGDLGKA-IPEGAVDNGQLRDVNEAIRSHAIETVGRKLREMMFRGMKQISSH-
```

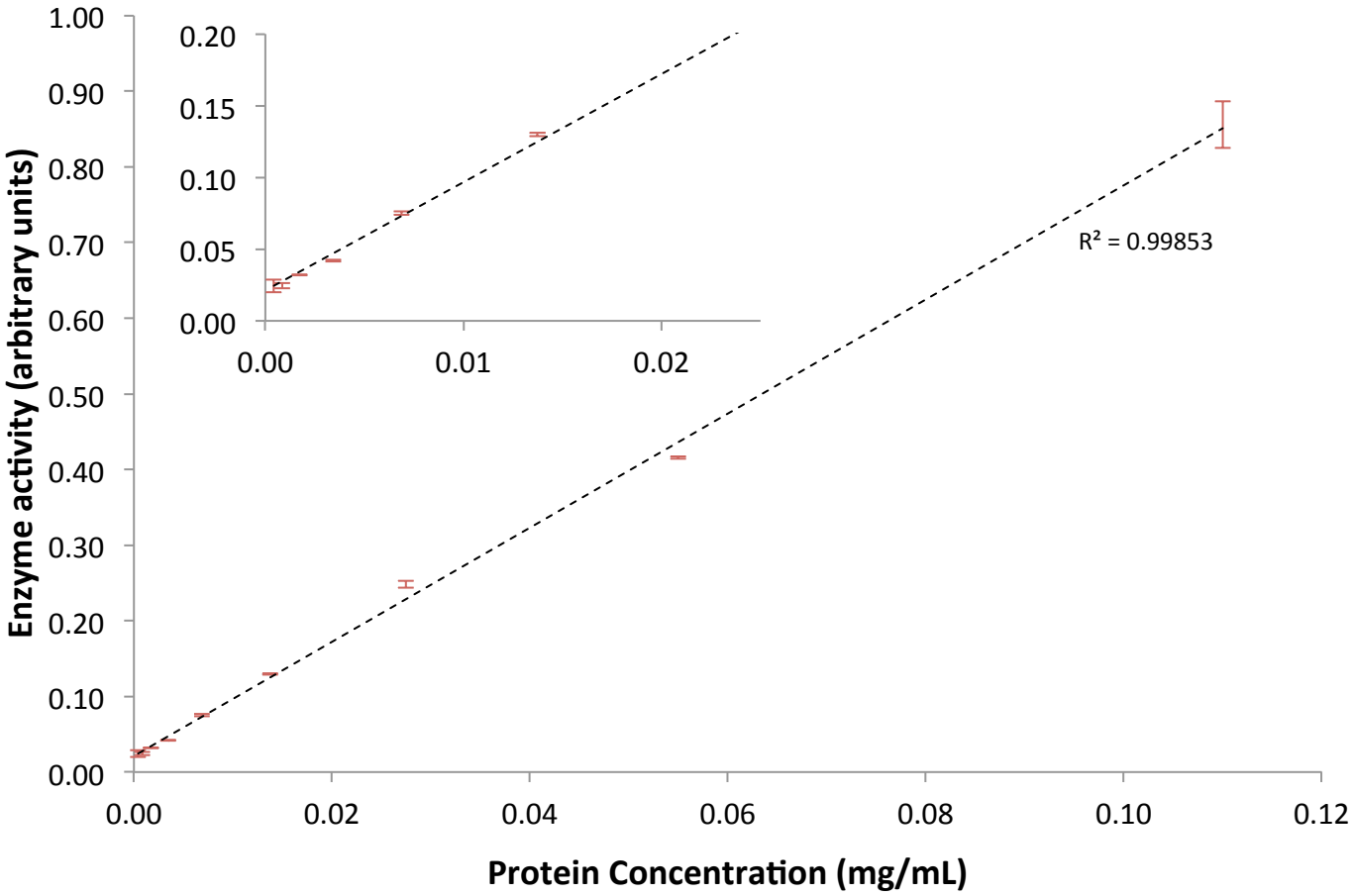
SI 4. An alignment of the sequences of Ia_KARI, Ec_KARI, and the four designed 2Ia_KARI constructs. Red text in the 2Ia_KARI constructs shows sequence insertions or deletions coming from Ec_KARI.

SI 5. Comparison between catalytic parameters on NADH and NADPH for selected KARIs.

Enzyme	K_M NADH [μM]	K_M NADPH [μM]	k_{cat} NADH [s^{-1}]	k_{cat} NADPH [s^{-1}]	k_{cat}/K_M NADH [$s^{-1}mM^{-1}$]	k_{cat}/K_M NADPH [$s^{-1}mM^{-1}$]	Catalytic Efficiency Ratio (NADH/NADPH)
Ia_KARI	< 1	< 1	2.6	3.3	>2,600	>3,300	0.8
2Ia_KARI-DD	< 1	< 1	1.7	3.0	>1,700	>3,000	0.6
2Ia_KARI-LT	< 1	< 1	0.8	1.0	>800	>1,000	0.8



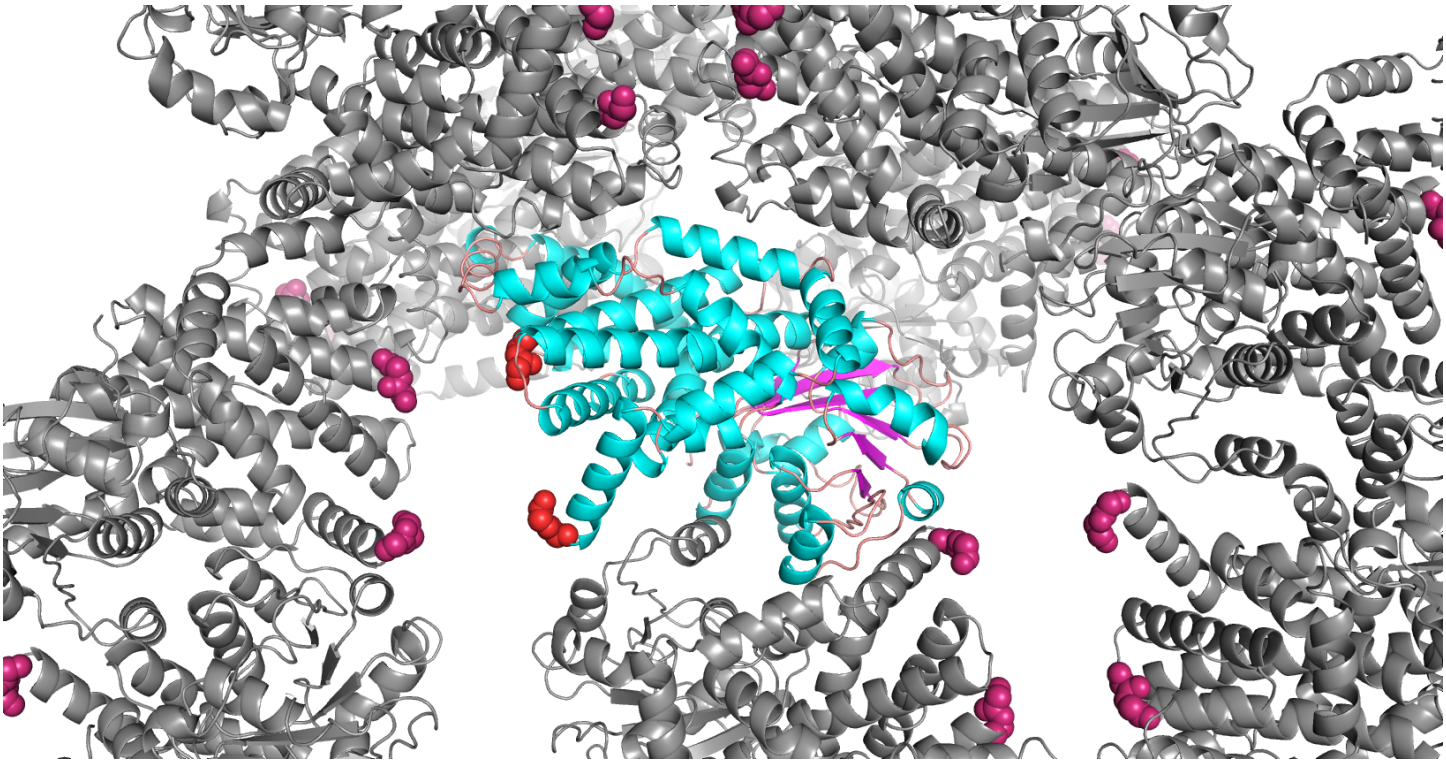
SI 6. Gel filtration chromatography analysis of 2Ia_KARIs. Gel filtration curves are shown for 2Ia_KARI-DD and 2Ia-KARI-LT, along with dimeric Ia_KARI WT and flanking controls (BSA and myoglobin). Inset shows proper power-law correspondence for predicted monomeric molecular weight.



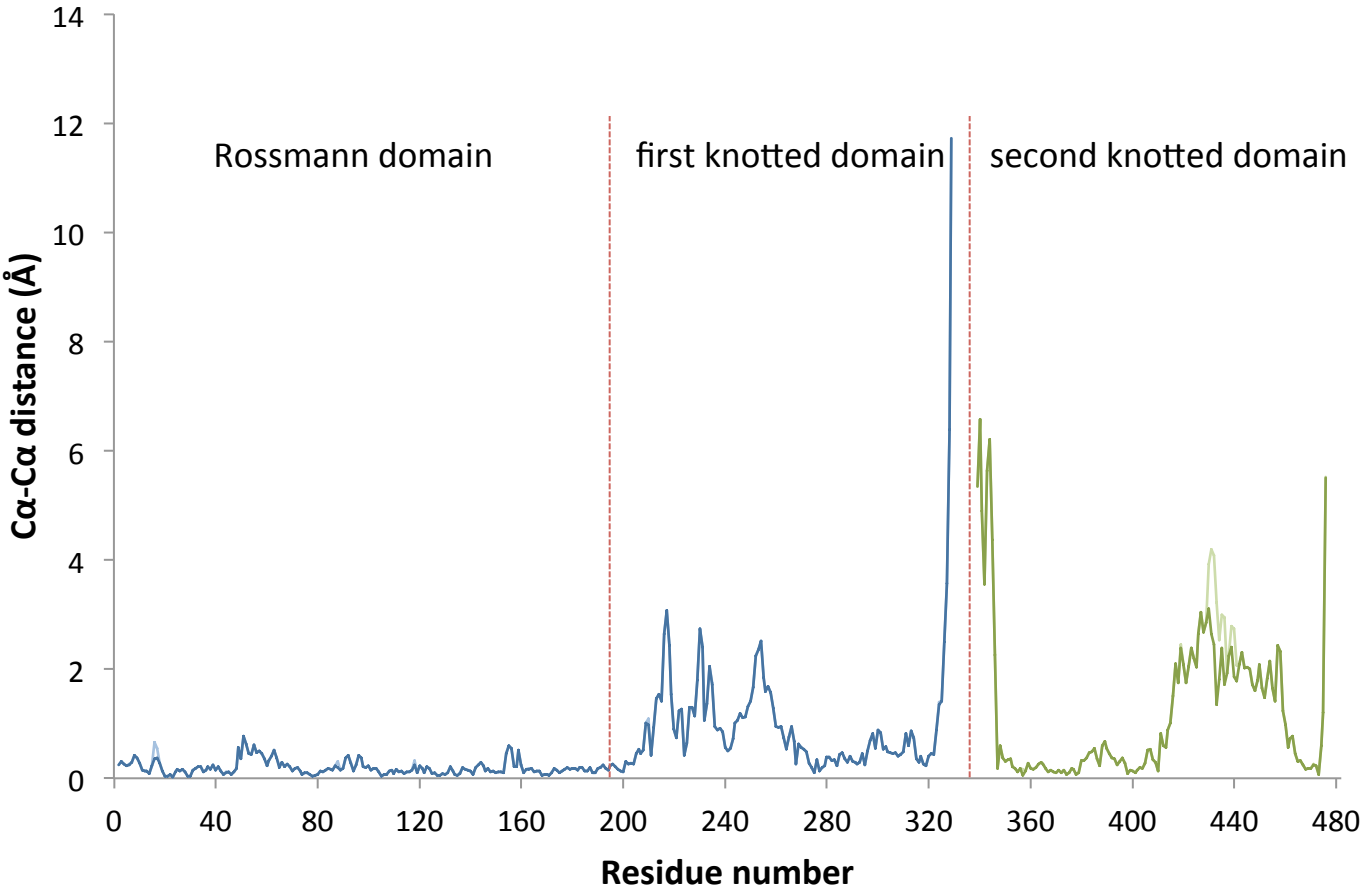
SI 7. Linear relationship between enzyme concentration and activity across a 512-fold range of dilutions for 2Ia_KARI-DD, showing monomeric behavior.

Data Collection	
Space Group	$P4_322$
a, b, c (Å)	103.9, 103.9, 142.1
α, β, γ (°)	90, 90, 90
Resolution (Å)	103.9 – 1.94 (1.99-1.94)
$R_{p.i.m}$ (%)	2.6 (22.2)
<i>/<σi>	7.39 (0.74)
Completeness (%)	99.2 (94.4)
Redundancy	10.3 (8.2)
Refinement	
No. reflections	
Rwork/Rfree (%)	15.8/17.9 (21.8/27.1)
No. atoms	
Protein	3753
Ligand	68
Water	237
RMSD	
Bond lengths (Å)	0.020
Bond angles (°)	1.128
Ramachandran Plot	
Favored	466
Allowed	14
Outliers	0

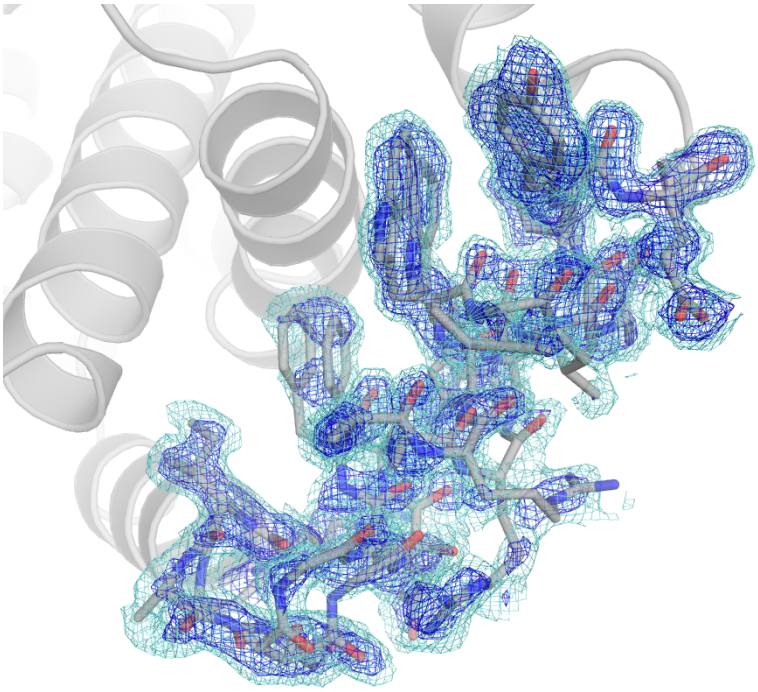
SI 8. Selected statistics on data collection and structure refinement for the structure of 2Ia_KARI-DD (RCSB 5E4R). Values in parentheses refer to the highest resolution shell.



SI 9. The packing of the obtained crystal of 2Ia_KARI-DD. A single protein chain (cyan and magenta) composes the asymmetric unit, and symmetry mates are shown in grey. Spheres represent the residues flanking the structurally unresolved interdomain connector.



SI 10. The Cα-Cα distances between 2Ia_KARI-DD and Ia_KARI. The lines are colored by which monomer of Ia_KARI is closer, blue for the first monomer and green for the second. When multiple Cα are present for a given residue, due to alternate conformations, the greater distance is shown in a lighter color. The residues between 330 and 339 are not structurally resolved.



SI 11. The α5+1-helix, showing two distinct conformations visible in the 2Fo-Fc map, contoured to 1.2σ (blue) and 0.65σ (cyan).